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Original Article

Evaluation of Flavonol Synthase (FLS) Gene Expression in *Cressa cretica* L. Using Plant Growth Regulators

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ABSTRACT

Background: *C. cretica* is a medicinal and ornamental halophytic plant which used for alterative, anthelmintic, tonic and aphrodisiac purposes. The purpose of this study was to detect the quercetin content in *C. cretica* and to evaluate the effects of exogenous application of abscisic acid and cytokinin hormones on FLS gene expression.
Methods: Abscisic acid and cytokinin (BAP) was sprayed in two-stages (8 days intervals) with two different concentration of 300 and 500 ppm. Quercetin content was confirmed by HPLC analysis in aerial parts of plant.
Results: The findings revealed that gene expression of FLS increased in the second stage of abscisic acid treatment. Observation showed significant differences in gene expression between treated and control samples, at level of 1%. In the first phase, there were no significant differences between the abscisic acid treated and control samples. In addition, the cytokinin treatment (500 ppm) resulted to the expression of the FLS gene at 5% level, which implied that cytokinin affected the FLS gene expression.
Conclusions: In this research, it was proved that the dihydroquercetin was present in *C. cretica* which changed to quercetin through the flavonol synthase enzyme.

Key words: Abscisic acid, Cytokinin, HPLC, ornamental plant, Quercetin

1. Background

Cressa cretica (*C. cretica*) is a perennial and ornamental plant from Convolvulaceae family, which is a valuable medicinal plant. *C.cretica* was traditionally used in medicine in India for alterative, anthelmintic, tonic and aphrodisiac purposes and is useful in leprosy, constipation, urinary discharges and asthma [1]. Furthermore, *C.cretica* used as green cover in lands with poor soil conditions and are naturally observed in salt marshes. Zia and khan [2] reported that *C. cretica* have a great economic potential as an ornamental plant for coastal saline areas [2].

Flavonol synthase (FLS) is an important enzyme of flavonoid pathway that catalysis formation of flavonols which formed from dihydroflavonols [3]. Flavonol synthase belongs to 2–oxoglotaratedioxygenase family. Flavonol synthase encoding gene has been

identified and sequenced in several plants [3]. A major product obtained from this gene is quercetin, which was used in combination with different compounds in order to produce various medicines. For instance, calendula ointment, eucalyptus syrup, cold tablets, echiherb, Prostatan, and Mellissa gel could be point out as some of the medicinal drugs which are derived from quercetin. Furthermore, Quercetin (pentahydroxy flavone) belongs to a large class of polyphenolic flavonoid compounds and are important natural antioxidants [4].

It has been demonstrated that external application of abscisic acid (ABA) increased the phenolic compounds and anthocyanin [5, 6]. Endogenous application of ABA has an important role in plant growth. For example abscisic acid generates and transmits signals to different parts of the plants when exposed to different stresses such as salinity, drought and cold conditions [7]. Moreover, abscisic acid initiates rapid ripening of seeds. It has been reported that ABA was involved in maturation-associated changes in grapes, which suggested that ABA played a significant role in triggering the flavonoid biosynthesis pathway [8].

FLS isoform (FtFLS2) from Tartary buckwheat was isolated and characterized by xiaohua [9]. The investigation suggested that FtFLS2 shared 48% identity and 67% similarity with the other FLS isoform that were previously reported (FtFLS1). HPLC was used to detect three main flavonolses in roots, leaves, stems, flowers and seeds of Tartary buckwheat. Furthermore, quantitative real time PCR was conducted to evaluate the expression levels. The mRNA accumulation of AcFLS increased in the leaves and flowers and reached its maximum level at 6-12 hours after wounding [10]. Identified open reading frame (ORF) region had 996 nucleotides that encoded 331 amino acid residues. Fang *et al*. (2013) studied the changes of total flavonolcontent, free flavonol content, and the two flavone contents during grape berry development. Results showed that quercetin, myricetin and three main flavonol (kaempferol, isorhamnetin and galangin) were detectable in this stage. It was reported that FLS activity had a highly positive correlation with the total contents of the flavonols. The analysis with immunoblotting detected two proteins who's the FLS activity was in accordance with signal intensity [11].

This study focuses on evaluation of FLS gene expression in *C. cretica* under exogenous application of two hormones. Our study is the first cultural and molecular work on *C. cretica* which investigated the effect of different exogenous application of abscisic acid (ABA) in two stages with two different concentrations. In addition, benzyl amino pourine (BAP) was applied in one stage. The expressed CcFLS cDNA level of *C. cretica* was determined in mature leaf tissue by using qPCR. The HPLC was conducted to illustrate the dihydroquercetin content in aerial part of plant.

2. Methods

2.1. Plant material and growth condition

Seeds from *C. cretica* were collected from the wild specimen plants randomly which it grew in zabol (Zabol, Iran. Longitude: 61 ° 32'0 ″ E and Latitude: 31 ° 0'0 ″ N). Seeds were sterilized with 10% bleaching water and were cultured in several pots. Two different methods were used for plant cultivation. The seeds were planted in the pots with a bed of sandy soil and grown at 20 °C with 12/12 (day/night) photoperiod. The grown seedlings were treated by spraying plant growth regulators (ABA and BAP) over them. The harvested samples were used

for RNA extraction of *C. cretica* leaves in response to treatments.

2.2. Plant treatment

Four month-old seedlings were treated with abscisic acid and cytokinin hormone in stable condition. The experiments were conducted as a completely randomized design with four treatments and three replications. Abscisic acid and cytokinin was sprayed in two-stages (8 days intervals) with two different concentration of 300 and 500 ppm. Treatments were applied on aerial part and in root growth medium of plant to ensure better hormone absorption. Tween 20 was used as a wetting agent in both hormone treatments and the control (water only) at concentration of 315 µl/l [12]. In order to ensure that the plant was influenced by hormones, the spraying was applied twice in the evening during two days. At the end of the experiment, samples were harvested, frozen in liquid nitrogen, and were stored at -80 °C until further use.

2.3. Quantification of Quercetin Aglycone

Leaves of *C. cretica* harvested and ground into a fine powder. 1 gram of powdered leaves were used to extract quercetin aglycone with 15 ml methanol/ water/acetic acid (85:15:0.5; v/v/v) in glass tubes. Extracted samples were vortexed for 30s, sonicated for 5 min and kept in dark chamber at room temperature. Extract (5 ml) was concentrated at 50 ºC using incubator set to remove solvent. Precipitation was dissolved in 5 ml of methanol (50%) containing 1.2 M HCl and sonicated for 5 min. Analysis of flavonol aglycone with HPLC was performed with the "UV-1575 intelligent UV-vis" detector (Jasco, Japan). UV visible spectra were reported in the range of 200 – 700 nm.

2.4. RNA extraction

RNA extracted from leaf tissue by using Cinna pure RNA extraction kit (SinaClon Bioscience, Iran). RNA concentration was tested by spectrophotometric absorbance at 260 and 280 nm of the samples in 10 mM Tris– HCl buffer, pH 7.5. RNA integrity was evaluated with 2% agarose gel analysis, stained with ethidium bromide and visualized with UV light. Prior to cDNA synthesis, extracted RNA was treated with DNase I to avoid contamination of genomic DNA.

2.5. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

cDNA was synthesized using 2-step RT-PCR kit (Vivantis, Malaysia) with oligo (dT) (18 bp) primer following manufacturer's instructions. cDNA was synthesized by using 10µg of total RNA, 40µM of oligo (dT), and 100 unit M-MuLV Reverse transcriptase. PCR mixture (25 µl) contained 2 µl of the cDNA template and 0.2 µM of both forward and reverse gene specific primers (Table 1).

Table1. PCR primers for RT-PCR and Real Time PCR (qPCR)

Gene name	Forward(5'→– 3')	Reverse(5'→– 3')
RT-PCR(For FLS)	CANCCAGCNATCACAAC	TACTCCTCATTCACTTCCC
qPCR(For FLS)	GAAATGATGGAGGCAGCAGG	ATTGGCCCAACTTCATGCTC
18s rRNA	GGACAGGATTGACAGATTGATAG	CTCGTTCGTTATCGGAATTAAC

N= A, T, C, G

2.6. qPCR analysis

cDNA was chosen as a template for quantitative Real Time PCR (qPCR) using the "Hot Taq Eva Green qPCR mix no ROX" (Cinnagen, Iran). qPCR was performed with RG 3000 sequence detection system (Corbett Research, CR, Germany). Cycle threshold (Ct) values were calculated for each sample based on a standard curve for each primer pair and then normalized to 18s rRNA. Real time RT_PCR was performed on FLS in three replicating in 96-well plate.

Serial dilution was prepared with different cDNA concentration to evaluate cDNA efficiency. Relative expression data was analysed based on the Livak comparative method. Serial corresponding amounts of cDNA templates were 1, 0.1, 0.01 and 0.001 µl. Results were graphically similar to the displayed reference values on abscissa as logarithms of DNA concentrations or CT-values. To facilitate the RT-PCR, the starting cDNA concentration for sets of experiment (e.g. 200 ng/reaction), was referred as 1 arbitrary unit. Ordinate units were CT values of the tested locus amplification.

3. Results

3.1. Morphological effects of abscisic acid and cytokinin

As the plant is a dicotyledonous, two petioles were formed at the beginning of its primary growth stage. At the next growth stage, stem grow rapidly compared to the root (Figure 1). Influences of ABA and cytokinine hormones on aerial and ground tissues of *C. cretica* were analysed separately. Abscisic acid caused twisted leaves and thinner petioles compared to the control plants. Treatment was most effective on terminal parts of leaves. In addition, terminal leaves were more prone to fall. Root growth was observed only at peripheral segments of the roots (Figure 1a). Cytokinin hormone proliferated leaves in a large amount. Furthermore, spaces between leaves diminished and production of peripheral branches improved compared to the control plant (Figure 1b). Application of this hormone enhanced development of roots through formation of numerous peripheral roots, and resulted in smaller leaf size in comparison to the control plants (Figure 1c).

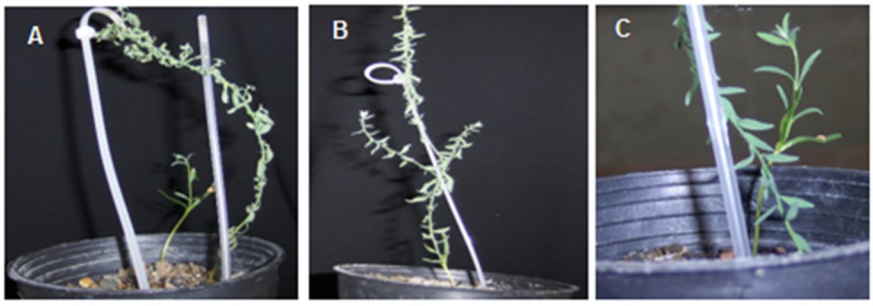

Figure 1. Morphological effects of abscisic acid and cytokinin hormones, **A**) Abscisic acid hormone, **B**) Cytokinin hormone, **C**) Control sample without treatment

3.2. Measuring 0f flavonol synthase gene activity and quercetin content

HPLC test was performed on aerial parts to investigate the presence of quercetin substance in plant. HPLC diagram verified that FLS gene synthesized flavonol synthase in *C. cretica*. This enzyme catalyzes the conversion of dihydroquercetin to quercetin. Results were then analyzed based on information within the peak area (Figure 2). Results proved that amount of quercetin available in aerial parts of *C. cretica* plant increased

compared to the control plant. All of these facts suggest that flavonol synthase (FLS) gene was active in aerial part of *C.cretica* and quercetin is present in it. This is the first report from flavonol synthase activity and production of quercetin in *C. cretica* plant through HPLC.

Table 2. Analysis of variance for FLS gene expression in C.cretica under ABA and BAP treatments

Source of variation	df	Mean squar
Treatments	3	1.304**
Experimental error	8	0.018
CV (%)		7.85
R- squar (%)		93.95

**: Significant at % 1 probability level

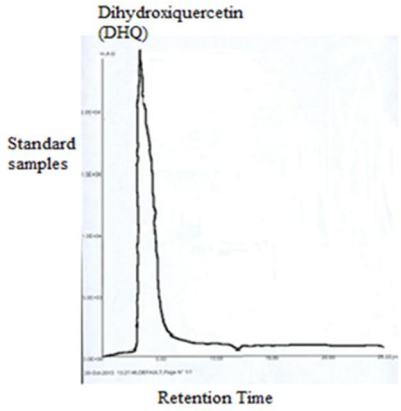

Figure 2. HPLC chromatogram of reaction products generatedby FLS enzyme assay of *C. cretica* extracts. Expressing FLS cDNS (CcFLS) using dihydroquercetin as a standard and substrate

3.3. RNA extraction and RT-PCR amplification

Total RNA was extracted from treated seedlings with ABA and BAP in two different developmental stages. RNA integrity was tested by 2 % agarose gel analysis (Figure 3a). Partial cDNA of FLS encodes a polynucleotide of 345 bp residues. Amplify fragments show high quality at an expected primer site (Figure 3). A separation primer was used to assess the gene quality. Based on the results, RT-PCR products show high quality (Figure 3b).

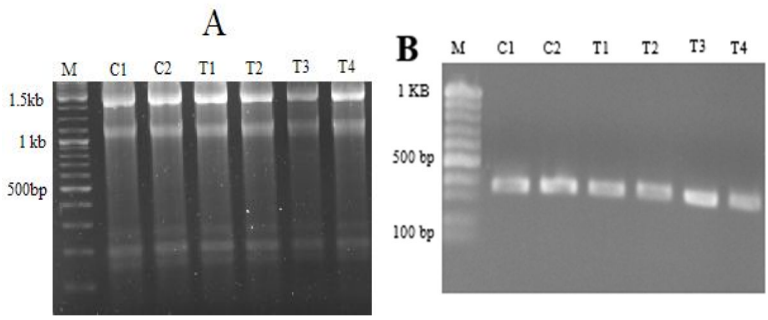

Figure 3. RNA extraction (**A**) and Qualitative analysis of Flavonol synthse gene expression using RT-PCR (**B**). C1: control for first stage of 300 ppm ABA; C2: control for second stage of 300 ppm ABA; T1: ABA treatment with 300 ppm concentration for the first stage; T2: ABA treatment with

300 ppm concentration in the second stage; T3: ABA treatment with 500 ppm concentration; T4: BAP hormone treatment with a concentration of 500 ppm

3.4. Quantitative gene expression of flavonol synthase

C_T and $\Delta\Delta C_T$ values were obtained from qRT-PCR results. One-way ANOVA (Table 2) and Duncan test (Table 3) was used to analyse obtained values statistically. Data revealed that there was a significant difference between the applied treatments in amount of FLS gene expression. The highest expression value for flavonal synthase was obtained for abscisic acid at 300-ppm concentration at secondary step. Furthermore, statistical analysis showed significant difference for flavonol synthase gene expression at 1% ($p<0.01$). Expression levels were also altered during first stage of treatment. However, obtained values were very low compared to the second stage. Results indicated that longer implementation of ABA treatment increased the overall expression of flavanol synthase compared to the control sample. Similarly, samples treated with cytokinin hormone significantly ($p<0.05$) increased gene expression of flavanol synthase (Table 3).

Table 3. Mean comparison of FLS gene relative expression levels under ABA and BAP treatments

Treatment	FLS
Control	0.953^{c}
300 ppm ABA first step	1.430^{b}
300 ppm ABA secondary step	2.397^{a}
500 ppm ABA first step	1.399^{b}
500 ppm BAP first step	1.635^{b}

Means within a column with the same letter are not significantly different ($P<0.01$)

Results show that the change mean of treatment 1 flavonol synthase gene expression was 1.399 in Treatment 1. This value increased as much as 1.45 units compared with the control sample. For second treatment (the second stage of abscisic acid treatment), the mean of gene expression was 2.397, which was 2.51 units further than control sample. This elevation was significant at the level of 1% ($p<0.01$). Third treatment was also similar to the first one, whose gene expression values is not very significant (Figure 4a).

Analysis of data showed that there is a significant difference in the value of FLS gene expression between the applied treatments. There is the highest expression value belonged to the second treatment where the abscisic acid was effective in development of gene expression. Furthermore, the lowest value of gene expression was related to Treatment 1 of the first stage of utilization of abscisic acid hormone. This comparison demonstrates that Treatment 1 had a higher expression compared with the control group. By spraying the abscisic acid hormone in the first stage, gene expression grew up as little as 0.45. The low value of flavonol synthase gene expression in the first stage in comparison with the second one may be due to the ageing stimulation property caused by the abscisic acid hormone. This property causes secondary metabolites including flavonoids and flavonols to be produced even further. The longer residence time of hormone in the plant tissue, caused the higher gene expression of these compounds (Figure 4b).

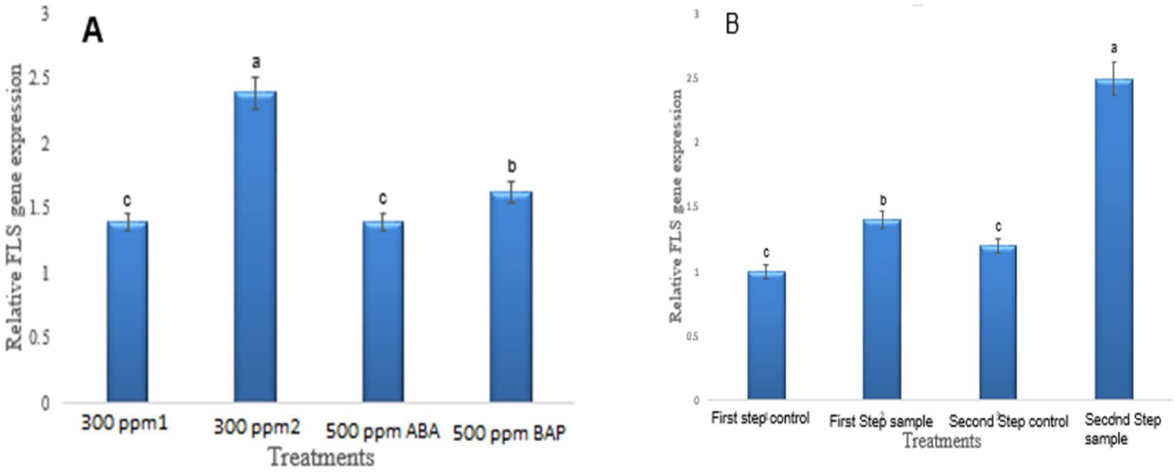

Figure 4. The expression of FLS gene in *C. cretica*.

Note: Hormone treatments including spray abscisic acid in two stages and each stage within 8 days at a concentration of 300 ppm and 500 ppm concentrations of abscisic acid (ABA) and cytokinin (BAP) single-stage tests. 300 ppm1 = treatment 1 and 300 ppm 2 = Treatment 2). (A) Effects of ABA treatment on FLS gene expression. (B) Comparison of FLS gene expression between first step and secondary step by 300 ppm ABA application. The relative abundance of each mRNA was normalized to the 18srRNA gene in the corresponding samples. Results are presented as relative expression against the transcript amounts of the corresponding gene

Results of using BAP hormone showed that this hormone has a significant effect on the expression of the scales gene. As shown in Figure 5, the concentration of 500 ppm of BAP hormone had a significant increase on FLS gene expression compared to ABA hormone (Figure 5a). The mean of changes on flavonol synthase gene expression was 1.635 for Treatment 4 (cytokinin hormone), with a 1.715 units increase compared with the control. For this treatment, mean was significant at 5% (Figure 5b).

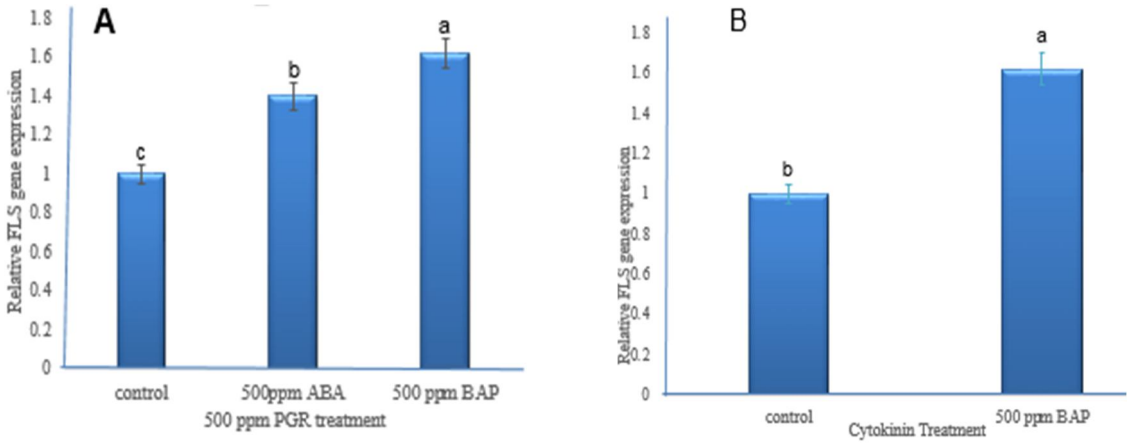

Figure 5. Comparison of quantitative expression between 500 ppm ABA and 500 ppm BAP application to the control sample (**A**). Effects of Benzyl amino purine (BAP= Cytokinin) treatment on FLS gene expression (**B**)

Analysis showed that there is a significant difference in the value of FLS gene expression between applied treatments. The relationship between gene expression of flavonol synthase and protein expression (containing quercetin

produced by gene expression) was obtained from the analysis of HPLC results in aerial parts of *C. cretica*.

In order to evaluate and analyse the transcripts amplified by PCR, it is required to ensure efficiency of PCR method and amplification of transcripts. Accordingly, it is required to calculate efficiency of sample propagations for both standard and obtained flavonol synthase genes. The amplification efficiency of these genes was obtained based on a standard curve. The regression lines equation and correlation coefficient (R^2) were 0.981 for flavonol synthase gene and 0.977 for standard 18srRNA gene. These figures prove that the amount of production doubles via each of the PCR amplification stages both theoretically and experimentally. In addition, the method of sample amplification via PCR confirmed linear relationship between CT value and precise amount of cDNA used for onset of reaction and amplification by primers. In other words, cDNA has been synthesized properly and its value has been accurate to be used in the reaction. All of these parameters confirmed a reliable and precise estimate of amplification efficiency for evaluating of quantitative flavonol synthase gene expression (Figure 6). Diagram shows the cycle number in horizontal axis and amount of absorbed fluorescent light in vertical axis. The number and interval of cycles increased when serial dilution decrease (Figure 6a). Serial dilutions (reductive volume of concentrations) are along the horizontal axis and the number of cycles is along the vertical axis. Amplification figure shows that for each serial, there is an acceptable interval of 3.3 cycles between each of the graphs (Figure 6b).

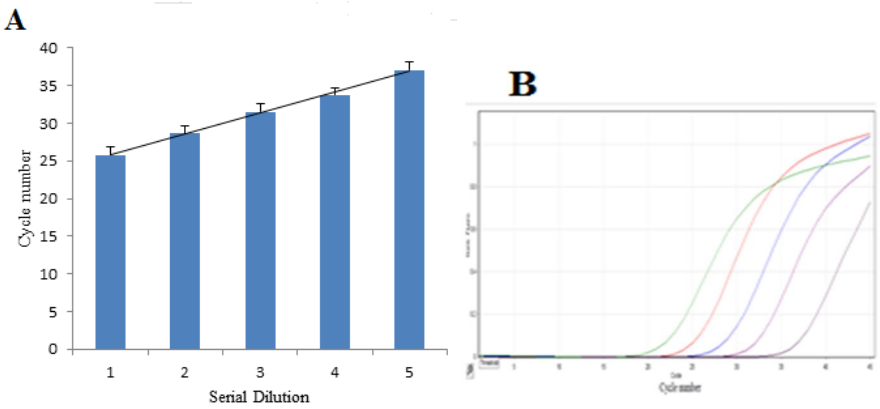

Figure 6. The schematic view of amplification curve drawn to achieve a standard curve for evaluating the gene expression value. (**A**) Diagram of serial dilution basis of slope; **B**) Amplification curve for serial dilutions

4. Discussion

4.1. Flavonol synthase gene activity and quercetin content

C. cretica herb, containing different medicinal compounds which can have an indirect and important role in human diet. In our study, to investigate expression of flavonol synthase gene in *C. cretica*, it was needed to determine the real presence of this compounds in plant. In this research, it was proved using HPLC that dihydroquercetin was present in the plant and changed into quercetin through flavonol synthase enzyme. Similarly, it was observed that flavonol synthase gene was active in aerial part and quercetin available in aerial part of plant. It is demonstrated that dihydroquercetin content by exogenous application of ABA

was higher than control. Shahat *et al*(2005), verified existence of five flavonoids compounds including quercetin in *C. cretica* using Spectroscopic methods (UV, FAB-MS, H-NMRand C-NMR) [13]. Liu *et al.*, introduced HPLC method as a reliable method to achieving the analysis of herbal extract and of flavonoids in plants. In this study for the first time, they confirmed the content of quercetin compound using RP-HPLC in the *Euphorbia helioscopia* plant [14]. In addition to this compound, Duenas *et al* reported the quercetinglucuronide using the HPLC-DAD-ESI/MS in the Green beans plant [15]. For the first time we find, presence of quercetin compound in *C. cretica* was proved through HPLC. Results obtained from HPLC at wavelength of 360 nm for control sample and one treated with abscisic acid hormone verified that ABA hormone resulted in increase of quercetin levels.

Our study indicated that the expression of flavonol synthase gene was definitely correlated with accumulation and aggregation of quercetin upon usage of abscisic acid hormone. It emerges that quercetin content is regulated through a special mechanism in mature and young leaves in every developmental stage of the plant. It is estimated that high amounts of quercetin within young and immature leaves protect the plant in early developmental stages. Therefore, it follows that the regulated and regulating factors of flavonol synthase gene are involved in developmental stages of *C. cretica*. Quercetin compound is valuable since it is present in many important drug formulations such as Melisan gel, Calendula ointment, Arnicle cream, Aphrodite tablets, Ekiherb tablet, and etc. As an important herb, the *C. cretica* can have a strong potential for treating many diseases and preparing raw materials for industrial and chemical medications thanks to containing quercetin and other unknown drug compounds.

4.2. RNA extraction from C. cretica leaves

Achieving RNA with high quality and quantity is the most critical stage in performing many molecular tasks. Extracting RNA fromplant tissue is very difficult, especially in the *C. cretica* plant, which grows like weeds and has many intervening compounds such as phenolic compounds and secondary metabolites. To do RNA extraction in the *C. cretica* plant, at first, method of Chomczynski and sacchi (1987) was applied using an RNA Plus extraction kit [16]. Result obtained from investigation of RNA extraction was not satisfactory on gel and biophotometer device. Kalinowska *et al* (2012) reported that the main problem are the time of RNA extraction regarding the phenolic compounds, oxidation and Quinone formation [17]. Aromatic compounds can delay the activities and applications of RNA by connecting to the RNA [18]. Methods related to RNA extraction from plants have numerous phenol-containing plants and secondary metabolites. Most of these methods are not applicable to plants, because they are costly and time-consuming and have no desirable results [16, 19, 20]. None of the used extraction methods exhibited successful extraction of RNA due to inability to remove secondary compounds. It seems that since manual approaches take long and is time-consuming, where the beginning and finishing times of extraction take many hours, and thus the RNA degrades during this period. As RNA lacks hydroxyl (OH) groups in carbon number 2, this has caused the RNA to degrade earlier. On the other hand, in long durations, probability of presence of RNase enzyme increases as a result of using different materials and instruments. Therefore, the mentioned reasons might account for the failure of these methods.

RNA was extracted hardly from *C. cretica* for the first time. Since no previous similar work had been done on this plant,

it was very challenging. Phenolic compounds and secondary metabolites interfere with purification and achieving proper RNA extraction because of having special properties. Accordingly, some artificial compounds and chemicals as silica particles and silica-containing membranes have been developed to deactivate these compounds. Silica particles are able to absorb RNA and prevent interference and connection of non-RNA compounds. This, in turn, enhances quality and purity of extracted RNA. In this research, in order to evaluate amount of flavonol synthase gene expression, quantity of extracted RNA was determined by a biophotometer device. To extract RNA utilized from Cinna Pure RNA Extraction Kit (sinnaclon). This kit connects to silica-containing membranes after washing and removing all excessive compounds and finally thins. Ding *et al.* (2008) proved that procedure of RNA extraction from plants containing phenolic compounds and secondary metabolites is desirable quality using silica particles [19]. Phenolic compounds and secondary metabolites seriously interfere with RNA extraction because of having some particular structural properties. There are many methods grounded on silica-containing gels which are able to purify plant RNA and remove interfering compounds [21]. Regardless of the method used for RNA extraction, it is necessary to measure the quantity of obtained RNA before evaluation of gene expression [22]. In this research, for the first time we managed to extract RNA from *C. cretica* with an appropriate quality and use them in molecular tasks of RT-PCR and Real Time PCR.

4.3. RT-PCR and qRT-PCR assay

Results of RT-PCR showed that amount of products in samples treated with abscisic acid hormone were more than control samples. Analysis of qPCR revealed a significance effect of ABA persistence on aerial parts. It was proved that 300 ppm concentration of ABA showed a remarkable quantitative influence on elevation of flavonol synthase gene expression. Table 2 shows that 300-ppm concentration of ABA in second stage (Treatment 2) and 500-ppm concentration of cytokinin hormone (Treatment 4) increased flavonol synthase gene expression. It was also seen that the second treatment and the first treatment had the highest and lowest impact on FLS gene expression, respectively. The first stage used ABA with a concentration of 300 ppm. Similar to our study, Amandeep *et al* proved effect of external application of ABA hormone on elevation of phenolic and anthocyanin compounds. They verified that the first stage of ABA application with 300 ppm concentrations increased phenolic compounds [12]. On the other hand, Li *et al.*, (2013), reported that abscisic acid had an inhibitory and reductive effect on expression of FtFLS1 and FtFLS2 genes, respectively [23]. In our study, results were contrary to the report given by [12]. It was observed that longer remains of ABA in plant, resulted great gene expression of flavonol synthase and quercetin content.

This study proved that the retention time of abscisic acid hormone in different aerial parts of *C. cretica* had a significant effect on increasing of gene expression and elevating concentration of important drug compositions. As hormone remains longer within plant, the time required for transmission of physiologic messages to different parts would be provided. It seems that abscisic acid plays important role in triggering of secondary metabolism. It may be case that in our research exposure time of plant to the tension of abscisic acid hormone along with concentration chosen for treatment has resulted in increased expression of FLS

gene. Furthermore, it is possible that since plants were grown and treated in pots within laboratory conditions, ABA hormone absorbed desirably resulting in increased genetic activity of FLS. It seems that [23] had investigated and treated plants grown under natural conditions. Since plants grown in outside environment are constantly subjected to a variety of biological and non-biological tensions, it is not possible to say with certainty that a specific treatment has resulted in increase, decrease, or expression inhibition of a certain gene.

To verify the results of RT-PCR and qPCR, confirming reaction efficiency is essential. A reliable method for ensuring accuracy of qPCR reaction is preparation of serial dilution. First, a serial dilution was prepared and then standard curve was drawn. Based on these, reaction efficiency was determined which confirmed results. The validity of experiments and reaction efficiency of both of FLS and 18srRNA genes were within standard range. Following the drawing of standard curve for each of these target and standard genes, it was observed that the slope of each of these serial dilutions was acceptable and within the 3.3 range (Figure 6). Therefore, replication of copies was done correctly by PCR. In another study, Liu and Saint used efficiency investigation method and standard curve drawing to confirm result of Real Time PCR. They reported that this method was able to determine efficiency of replication of each cycle. Validity of used method confirmed existence of an intrinsic relationship between initial values of genetic copies and kinetic parameters [24]. Stuart *et al* also used efficiency determination method for validation of experiments and for quantitative analysis studies. They reported that difference between results of relative and accurate quantitative analyses has relation through calculated efficiency difference. Through calculation of replication efficiency for analyzed samples, it is possible to compare results without drawing a standard curve [25].

5. Conclusions

The finding of this research suggest that external application of ABA and BAP (Cytokinin) treatments can be used to increase the expression of FLS biosynthetic pathway genes in the leaves and probably in flowers and seeds of *C. cretica*. We advise other researchers to use this plant for pharmaceutical use and the extraction of pharmaceutical products. The identification and discovery of genes associated with drought tolerance and salinity can also be applied in breeding programs. The clonning of resistant genes can make plants susceptible to unsuitable conditions of environment.

Abbreviation

FLS: Flavonol synthase; C.cretica: Cressa cretica;. BAP: Benzyl Amino pourin;. ABA: Abscisic Acis; PPM: Part Per Million; HPLC: High Performance Liquid Chromatography; qPCR: Quantitative PCR; CT: Threshold Cycle. cDNA:Complementary DNA; RT-PCR: Reverse Transcriptase PCR.

Conflict of interests

The authors have no conflicts of interest to declare.

Authors contribution

Study conception and design: D. N. Interpretation of the data and critical revision of the manuscript for important intellectual content: M. S. Interpretation of the data and statistical analysis: B.A. F.

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